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Characterisation of nerve-mediated ATP release from detrusor muscle; pathological implications

Short title: Detrusor nerve-mediated ATP release

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Abstract.

Background and Purpose.

To characterise the molecular mechanisms that determine variability of atropine-resistance of nerve-mediated contractions in human and guinea-pig detrusor smooth muscle

Experimental Approach.

Atropine-resistance of nerve-mediated contractions, and the role of $P2X_1$ receptors, was measured in isolated preparations from guinea-pigs and also humans with or without overactive bladder syndrome, from which the mucosa was removed. Nerve-mediated ATP release was measured directly with amperometric ATP-sensitive electrodes. Ecto-ATPase activity of guinea-pig and human detrusor samples was measured *in vitro* by measuring the concentration-dependent rate of ATP breakdown. The transcription of ecto-ATPase subtypes in human samples was measured by qPCR.

Key Results

Atropine resistance was greatest in guinea-pig detrusor, absent in human tissue from normally-functioning bladders and intermediate in human overactive bladder. Greater atropine resistance correlated with reduction of contractions by the ATP-diphospho-hydrolase apyrase, directly implicating ATP in their generation. E-NTPDase-1 was the most

abundantly transcribed ecto-ATPase of those tested and transcription was reduced in tissue from human overactive, compared to normal, bladders. E-NTPDase-1 enzymatic activity was inversely related to the magnitude of atropine resistance. Nerve-mediated ATP release was continually measured and varied with stimulation frequency over the range 1-16 Hz.

Conclusion and Implications

Atropine-resistance in nerve-mediated detrusor contractions is due to ATP release and its magnitude is inversely related to E-NTPDase-1 activity. ATP is released under different stimulation conditions compared to acetylcholine that implies different routes for their release.

Key words: Detrusor smooth muscle; atropine resistance; ATP release; ecto-ATPase activity.

Abbreviations:

ABMA	α,β-methylene ATP;
ACh	acetylcholine;
ARL-67156	6-N,N-diethyl-D-β,γ-dibromomethyleneATP;
EFS	electrical field stimulation;
E-NTPDase	ecto-nucleoside triphosphate diphosphohydrolase;
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid;
human-DO	tissue from patients with detrusor overactivity;
human-stable	tissue from patients with normal bladder function;
IDO	idiopathic detrusor overactivity;
NDO	neuropathic detrusor overactivity;
TTX	tetrodotoxin.

Bullet point summary

What is already known

- Atropine resistance in detrusor results from nerve-mediated ATP release.
- Atropine resistance when it occurs in human detrusor is from patients with overactive bladder pathologies.

What this study adds

- Atropine resistance results from incomplete hydrolysis of ATP at the nerve-muscle junction.
- Nerve-mediated ATP release occurs at a lower stimulation frequency range that generate contractions

Clinical significance

- With humans, functional nerve-mediated ATP release is associated with overactive bladder.
- Selective modulation of nerve-mediated ATP offers a drug target to manage overactive bladder.
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Introduction

Contraction of urinary bladder detrusor smooth muscle is initiated by excitation of postganglionic parasympathetic fibres that release acetylcholine (ACh) and ATP. With human detrusor from normal bladders ACh is the sole functional transmitter as atropine completely abolishes nerve-mediated contractions (Bayliss *et al*., 1999). However, with detrusor from most other mammals, part of the contraction is atropine-resistant. Atropine-resistant contractions are proposed to be mediated by ATP acting on $P2X_1$ receptors (Lee *et al*., 2000) as they are greatly attenuated by the non-hydrolysable analogue of ATP, α,β-methylene ATP (ABMA: Palea et al, 1993; Peterson and Noronha-Blob, 1989) by rapid desensitisation of the receptor (North and Surprenant, 2000). In addition, ATP release is associated with nerve-mediated detrusor contractions (Burnstock *et al*., 1978; Hashitani and Suzuki, 1995).

However, some studies propose that ATP also activates other P2X receptors, in particular a $P2X_{1,4}$ heteromer (Kennedy *et al*., 2007; Syed and Kennedy, 2012).

With human detrusor, atropine-resistance of nerve-mediated contractions occurs in particular with advancing age (Yoshida et al., 2001; but see Yokota *et al*., 1996) and with overactive bladder symptoms accompanying several pathologies, including neurological injuries, outflow tract obstruction and idiopathic causes (Bayliss *et al*., 1999). Similar pathologies in animal models also increase the proportion of the purinergic component of nerve-mediated contractions (Moss *et al*., 1989; Mumtaz *et al*., 2006).

One area of the study was to determine why atropine-resistance varies between species (human and guinea-pig) and why it occurs more in functional bladder pathologies. Several hypotheses may be proposed for this variability: i) the potency and efficacy of $P2X_1$ agonists to generate responses may vary with detrusor from different species and pathologies; ii) detrusor not exhibiting atropine-resistant nerve-mediated contractions may not release ATP; iii) nerve-mediated ATP release always occurs but is variably hydrolysed in the nerve-muscle junction by ectoATPases and in some detrusor preparations may not activate the detrusor muscle. A nucleotide-specific group of ecto-ATPases is the ecto-nucleoside triphosphate diphospho-hydrolases (ENTPDases), which have eight paralogues (Zimmerman *et al.*, 2012).

Further insight into how ATP-dependent contractions may be selectively manipulated comes from evidence that ATP and ACh may be released from postganglionic nerve terminals by different pathways. Indirect evidence suggests that ATP is released at smaller stimulation rates, compared to ACh (Calvert *et al*., 2001; Pakzad *et al*., 2016, Chakrabarty *et al*., 2019). There is more direct evidence that the PDE-5 inhibitor sildenafil abolishes nerve-mediated ATP release (Chakrabarty *et al*., 2019), however, this should be set against the observation

that ACh is also modulated by agents such as adenosine (Silva-Ramos *et al*., 2015). The development of amperometric ATP-selective electrodes potentially allows for the real-time measurement of nerve-mediated ATP release in detrusor muscle. Measurement of the frequency-dependence of ATP was attempted in his study to relate it to the above contractile data that suggests frequency-dependence of release of different neurotransmitters.

The motivation for the study was to elucidate how atropine-resistant, ATP-dependent contractions are generated. As these are a feature of detrusor overactivity in the human bladder, this should provide targeted drug models to attenuate specifically this particular bladder pathology.

Methods

Ethical approval and tissue sources. Human and guinea-pig detrusor was used. Human biopsies were obtained at open-surgery from patients with idiopathic (*n*=5; 50±15yr; IDO) or neuropathic (*n*=6; 34±10yr; NDO) detrusor overactivity (human-DO), or those undergoing cystectomy with no DO symptoms (human-stable, *n*=9; 57±14yr). All procedures were in accordance with ethical committee approval of University College London Hospitals, and the 1964 Helsinki declaration. NDO and IDO data were not significantly different in any variable and were merged. Patient ages of the merged DO and the stable groups were not statistically different. Biopsies were brought to the laboratory in ice-cold Ca^{2+}-free Tyrodes within one hour of excision and used immediately. Animal care and experimental procedures were in compliance with the University of Bristol Ethics Committee (approval 17.09.2014) and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and studies reported in compliance with the ARRIVE guidelines (McGrath and Lilley, 2015; Curtis *et al.* 2015). Adult guinea-pigs were used as detrusor function has been previously well-characterised. Animals (Dunkin-Hartley, males, 350-400g) were procured by the local animal services unit, University of Bristol, housed singly in straw-floored cages at 22°C with a 12hr light-dark cycle and with water and food available *ad libitum*. Animals were killed by a Schedule 1 procedure; by injection with Na pentobarbital (200 $mg.kg^{-1}$, i.p.) and cervical dislocation, verified by a lack of corneal and spinal reflexes, and the bladder immediately removed through a laparotomy.

The mucosa (urothelium and lamina propria) was removed from human and animal tissue, and detrusor strips (<1 mm diam, 5 mm length) dissected, in Ca^{2+}-free Tyrode's solution, for tension and ATP release experiments. The remainder of the tissue was cut into three or four

pieces (≈20 mg each) with a fresh sharp razor blade and frozen in liquid-N_2 for RNA extraction and ATPase activity measurement.

Tension recording, nerve-mediated ATP-release and measurement of intracellular Ca^{2+}. Detrusor strips were tied to an isometric force transducer and a fixed anchor in a horizontal trough and superfused with Tyrode's solution at 4 ml.min^{-1}. Electrical field stimulation (EFS), *via* Pt plates in the sides of the trough, used 0.1 ms pulses in 3-s trains (frequency 1-32 Hz) every 90-s. Concentration-response curves for ATP and ABMA were constructed with unstimulated preparations, using test concentrations between 10^{-6} to 2.10^{-2} M for ATP and 10^{-8} to 10^{-4} M for ABMA in equal half-log increments. Force-frequency or concentration-response curves were fitted to equation 1:

$$T = (T_{max}x^n)/(x^n + x_{1/2}^n) \qquad 1)$$

T_{max} is the maximum force at high frequencies or concentrations, x; $x_{1/2}$ the frequency ($f_{1/2}$) or concentration (EC_{50}) required to elicit $T_{max}/2$; n is a constant. To measure the effect of ABMA (1 µM) on nerve-mediated contractions: EFS was stopped after control recordings; ABMA was added to the superfusate and after relaxation of the resulting contracture EFS recommenced (about 15 minutes); ABMA was then washed out. For atropine (1 µM) or apyrase (10 U.ml^{-1}) the agent was added to the superfusate, with the preparation stimulated throughout at 8 Hz.

Amperometric ATP electrodes (Sarissa Biomedical Ltd, Coventry, UK) were used to measure nerve-mediated ATP release with the active tip (2 mm length, 50 µm diameter) placed on the surface of the preparation parallel to the longitudinal axis. A null electrode, lacking the sensing surface was similarly placed and both polarised to 0.65 V. Electrode outputs formed the inputs to a home-made differential amplifier with high common mode rejection, and the output recorded to attenuate stimulation artefacts. Tyrode's superfusate contained 2 mM

glycerol, required for the enzymatic detection of ATP. Prior to experiments the system was calibrated by exposure to 10 µM Na_2ATP – electrodes had a linear response between 0 and 10 µM ATP (see Figure 1A). ATP transients were elicited by EFS (1-24 Hz). Two ATP/force-frequency relationships at 20-min intervals were done as time controls of the percentage of second compared to the first calculated. At 8Hz: tension 101.4±10.6%; ATP 100.2±15.3%, at 12 Hz: tension 102.2±11.9%; ATP 98.8±11.6% (*n*=5).

In a separate series of experiments to measure nerve-mediated release at a fixed frequency (8 Hz), superfusate samples (100 µl) were taken at a fixed distance (1 mm) above the preparation and 2-s after initiation of stimulation and analysed by a luciferin-luciferase assay as described previously (Kushida and Fry, 2016).

Myocytes were isolated from detrusor strips by collagenase dispersion. Fura-2 AM (5 µM) was added to cell suspensions for recording intracellular [Ca^{2+}] during control periods an on exposure to ABMA. The cell preparation procedure, experimental protocol and signal calibration have been explained in detail (Montgomery and Fry, 1992; Wu and Fry, 2001); all experiments were at 36°C.

Gene expression of ENTPDases. Total RNA was extracted from frozen tissue (30 mg) using an RNeasy Mini Kit (Qiagen, UK) as per manufacturer's instructions. RNA integrity was determined with an Aligent 2100 bioanalyser using the 18S and 28S ribosomal RNA bands as controls. This clearly showed visible single peaks indicative of high-quality RNA: the RNA concentration of each sample (7 µl) was determined with a Genequant 1300 spectrophotometer (VWR, UK). cDNA was synthesised from each RNA sample and then used for qPCR reactions using specific primers for ENTPDase-1, -2, -3, -5. ENTPdase-1, -2 and -3 were chosen as they are extracellular enzymes, ENTPDase-5 although intracellular, may be

secreted (Zimmermann *et al*., 2012). The resulting amplified RT-PCR products (TaqMan system, ThermoFisher Scientific) were separated by 1.5% agarose gel electrophoresis and visualised with SyberGold (Molecular Probes), quantified and expressed as a proportion of 18S cDNA.

Measurement of ecto-ATPase activity. Frozen detrusor samples (four 20-mg samples per bladder or biopsy sample) were separately thawed in 3 ml Ca^{2+}-free HEPES Tyrode's, then equilibrated at 37°C in 3 ml Tyrode's for 30 min. Samples were then transferred to 980 µl Tyrode's, two of which contained the ENTPDase inhibitor ARL-67156 (100 µM). After a further 10 mins, 10 mM Na_2ATP stock (20 µl in Tyrode's) was added for a final [ATP] of 0.2 mM. Subsequently, 10 µl aliquots were added to 1.99 ml Ca^{2+}-free HEPES Tyrode's with 5 mM EDTA at 0, 5, 10, 20 and 30 mins for ATP analysis by a luciferin-luciferase assay (GloMax 20/20, Promega, UK): the initial rate of ATP breakdown was calculated. After 30 mins the samples were washed in 3.5 ml Tyrode's and the ATP breakdown rate at 0.5 mM initial [ATP] re-commenced, the cycle was repeated for initial [ATP] of 1.0, 2.0 and 5.0 mM. A final run in 0.2 mM initial [ATP] was done, the initial rate was compared to the first estimate and used if within 10%. Finally, tissue samples were weighed and assayed for protein content (Bradford Assay, ThermoFisher Scientific). ATP concentrations at all time- and concentration-points in the presence of ARL-67156 were subtracted from those in its absence and used as the ENDTPase (ARL-dependent) rate. Initial rate, V_{ATP}, was plotted as a function of starting [ATP] to estimate the maximum rate at high concentrations, V_{max}, and the K_m of the reaction: $V = (V_{max}[ATP])/(k_m + [ATP])$.

Data presentation and analyses, experimental design. Most data are means±SD (*n*=number of separate human biopsies or guinea-pig bladders). Data for the effects of atropine and

apyrase are expressed as medians [25,75% interquartiles] as they were highly skewed sets in some instances. Data sets were compared by ANOVA followed by *post-hoc* Fisher's least significant difference comparison only if *F* was significant and there was no variance inhomogeneity: a value of $p<0.05$ (*) was accepted as significantly different. No data outliers were excluded. KaleidaGraph (RRID:SCR_014980) was used for data analysis and curve-fitting with a non-linear iterative fit program. Sample size calculations (*www.3rs-reduction.co.uk*) used previous experimental data (Harvey *et al*.,2002; Pakzad *et al*., 2016) with animal and human tissue suggested group sizes of *n*=5-6 for 80% power and 0.05 for a type-I error. Data and statistical analyses comply with BJP's recommendations and requirements on experimental design and analysis (Curtis *et al.* 2018). Individual data points to compile summaries in Table 1 and Figure 3 are shown in Supplement 1 (Figures S1a-i). Experiments were either interventional or compared data from either normal or pathological human bladder samples and no randomisation or blinding of samples was undertaken.

Materials: Tension and ATP release experiments were at 37°C in Tyrode's solution (mM): NaCl, 118; KCl, 4.0; $NaHCO_3$, 24; NaH_2PO_4, 0.4; $MgCl_2$, 1.0; $CaCl_2$, 1.8; glucose, 6.1; pyruvate, 5.0; gassed with 95%O_2,5%CO_2 (pH 7.45±0.03). Ca^{2+}-free Tyrode's solution contained HEPES (10 mM) + NaCl (14 mM) to replace $NaHCO_3$, pH 7.4 with 1M NaOH and gassed with 100%O_2. Atropine, apyrase, ABMA, tetrodotoxin (TTX), ARL 67156 and carbachol were stored as aqueous stocks and added to Tyrode's for appropriate final concentrations. All chemicals were from Sigma, UK.

Nomenclature of targets and ligands. Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and

are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos, et al., 2017, Alexander, Fabbro, et al., 2017, Alexander, Peters, et al., 2017).

Results

ATP-dependence of nerve-mediated contractions. EFS contractions in human and guinea-pig preparations were abolished by TTX (1 μM); in addition, contractions from human-stable bladders were also completely abolished by atropine (1 μM). However, with preparations from overactive human bladders (human-DO) and guinea-pigs atropine did not completely abolish contractions, leaving an atropine-resistant component: the absolute tension values and the percentage of the contraction remaining are shown in Table 1 (and Supplement 1; Figure S1a,b). Involvement of ATP as a neurotransmitter was indicated in four ways: i) ABMA (1 μM) abolished atropine-resistant contractions (not shown: see also Bayliss *et al*. 1999; Peterson and Noronha-Blob, 1989); ii) the ectoATPase inhibitor ARL 67156 increased guinea-pig detrusor contractions to 1.42±0.15 times control (p<0.05 n=5), and reduced $f_{1/2}$ values (5.5±0.7 to 4.8±0.9 Hz, p<0.05, n=5); iii) pre-treatment with ABMA reduced significantly contractions from human-DO and guinea-pig detrusor, but not from human-stable bladders (Table 1 and Supplement 1, Figure S1c); iv) the non-specific ATPase, apyrase had no effect on detrusor contractions of human-stable bladder but reduced those from human-DO and guinea-pig bladders (Table 1 and Supplement 1; Figure S1d). Moreover, the percentage reduction by apyrase was similar to the atropine-resistant percentage in the three groups.

The reason for variable atropine resistance in the three cohorts may be explained by: i) the potency and efficacy of agonists at detrusor $P2X_1$ receptors are different; ii) the amount of

ATP released by motor nerves varies; iii) ATP is hydrolysed to varying extents at the neuromuscular junction. These possibilities were subsequently tested.

Potency and efficacy of ABMA. Contractions from detrusor strips in the absence of EFS, and intracellular Ca^{2+} transients from isolated myocytes generated by addition of ABMA to the superfusate were recorded. With detrusor strips and isolated myocytes the pEC_{50} values were not significantly different between the three groups (Table 1, and Supplement 1; Figure S1e). The efficacy of 1 μM ABMA to generate tension was also similar between the groups (Table 1, and Supplement 1; Figure S1e). Thus, variability of atropine-resistance between the three groups cannot be due to differences of detrusor responsiveness to ABMA. It has been reported that ATP may act *via* receptors in addition to $P2X_1$ (Kennedy *et al*., 2007), the latter desensitised by ABMA. This was tested in six human samples (three with idiopathic DO and three normal) by adding ATP (1 mM) before and after exposure to ABMA. The response after ABMA was 6.5±2.5% of that before (with no difference in the values obtained with normal or DO samples) and suggests that a small fraction of the response to ATP is *via* a subtype other than $P2X_1$.

Nerve-mediated release of ATP. Real-time ATP release was measured with amperometric ATP electrodes during EFS (1-24 Hz for all interventions) from guinea-pig preparations: Figure 1A shows an example of ATP-electrode and tension recordings from a preparation stimulated, in this case, at 2 Hz. Electrode responses are from ATP and null electrodes, with the differential recording (ATP-null) used for analysis. The arrows at the peak of the tension and ATP-null traces marks the time to maximum response, where the tension peak always preceded the ATP-null response (see Discussion). The inset of Figure 1A shows an example of an ATP calibration pulse with a linear calibration curve constructed for several ATP concentrations.

ATP-electrode responses were unaffected by the muscle contraction itself as no response was elicited with 10 µM carbachol added to the superfusate (*n*=7; Figure 1B).

EFS-induced contractions and ATP transients were abolished by 1 µM tetrodotoxin (*n*=9, Figure 1C, 8 Hz in this example). ATP transients were unaffected by 1 µM atropine in magnitude or duration (11.1±1.4 and 10.3±2.1 s, without or with atropine, *n*=6, p>0.05), although contractions were reduced (*n*=6, Figure 1D, 4 Hz in this example). Nerve-mediated ATP release, in the presence of ARL 67156 to reduce ATP breakdown, was also measured in the three cohorts (guinea-pig; human-stable; human-DO) at a fixed frequency (8 Hz) using a luciferin-luciferase assay: values were not significantly different between each other: 0.81±0.41; 1.03±0.57; 0.62±0.30 pmol.µl^{-1} (ANOVA, *p*>0.05, all *n*=5).

ectoATPase activity and E-NTPDase expression. In contrast to ABMA, ATP exhibited a variable potency on detrusor contractions in the different cohorts: the ATP pEC_{50} was smaller in human stable preparations than in human-DO and guinea-pig preparations (Table 1, and Supplement 1; Figure S1f). This may be due to a differential hydrolysis of ATP in the nerve-muscle junction. This was tested by measuring ecto-ATPase activity in human and guinea-pig detrusor tissue, as well as gene expression for extracellular ATPases (E-NTPDases) in human detrusor from stable and overactive bladders.

Detrusor samples showed ATPase activity that was partially reduced by ARL 67156 (Figure 2A, sample experiment from human-stable preparation). Reactions were analysed by calculation of V_{max} and k_m values for total ATPase activity, as well as the ARL 67156–dependent fraction: the latter was used as an estimate of ecto-ATPase activity. The V_{max} for the ARL 67156–dependent component (V_{max}-ARL) was significantly greater in detrusor from human-stable bladders, compared to those from human-DO bladder and from guinea-pig bladder; these

latter were not significantly different (Table 1, and Supplement 1; Figure S1g). K_m values for total (K_m-total) and ARL-dependent (K_m-ARL) ATPase activities were similar for all three groups. Furthermore, V_{max}-ARL and K_m-ARL values for detrusor from idiopathic (IDO, *n*=5) and neurogenic (NDO, *n*=6) were similar; the merged data set is shown in table 1 (IDO *vs* NDO: V_{max}-ARL: 0.66±0.19 *vs* 0.55±0.12 nmol.mg $prot^{-1}.s^{-1}$, *p*>0.05; K_m-ARL: 1.63±0.25 *vs* 1.52±0.79 mM, *p*>0.05).

The dependence of percentage atropine resistance or reduction by apyrase on $V_{ATP,max}$-ARL is shown in Figure 2B. Thus, human-stable bladder has no ATP-dependent component of the nerve-mediated contraction (no atropine resistance or reduction by apyrase) and the highest V_{max}-ARL value, guinea-pig detrusor is at the other end of the spectrum with human-DO detrusor in an intermediate position.

RNA expression of four ecto-ATPase subtypes (ENTPDase-1, -2, -3 and -5) was measured in tissue from human-stable and human-DO bladders. ENTPDase-1, -2 and -3 are extracellular enzymes, ENTPDase-5 whilst intracellular may be secreted and so was included (Robson *et al*., 2006; Zimmermann *et al*., 2012). ENTPDase-1 was expressed most in human tissue, but expression was significantly less in tissue from overactive bladders (Table 1, and Supplement 1, Figure S1h), consistent with reduced ecto-ATPase activity. Consistent with variable ATPase activity in the three cohorts is that ATP dose-response curves for contracture development showed different pEC_{50} values (Table 1). Thus, the pEC_{50} was smallest in human-stable detrusor, greatest in guinea-pig tissue and intermediate in human-DO tissue.

Frequency-dependence of nerve-mediated ATP release. ATP transients were recorded in guinea-pig detrusor by EFS using 3-s trains of stimuli at frequencies from 1-24 Hz (Figure 3A). The magnitude of the ATP transients increased with stimulation frequency but reached a

maximum at lower frequencies than did contractions. Thus, tension and ATP transients showed different $f_{1/2}$ values ($f_{1/2}$ = frequency for half-maximal response, Figure 3B); tension 7.8±2.0 Hz (n=18) *vs* ATP 2.2±1.0 Hz (n=6, p<0.05). At higher frequencies the contour of the ATP transient also changed in some experiments, with a distinctive tail and the peak amplitude sometimes diminishing. Thus, the integral of the ATP transient over 10 seconds ($\int ATP_{10}$) was also calculated and the $f_{1/2}$ values again estimated: $f_{1/2}$ values for the ATP amplitude and $\int ATP_{10}$ were not significantly different (2.2±1.0 *vs* 3.4±0.8 Hz, p>0.05, n=6). In the presence of atropine the force-frequency curve shifted to the left and was similar to the ATP-frequency curve ($f_{1/2}$ values: 3.6±1.4 Hz vs 2.2±1.0 Hz, p>0.05 – individual data values in Supplement 1; Figure S1i). This is consistent with the hypothesis that the tension-frequency curve in the presence of ATP is determined by nerve-mediated ATP release.

Discussion

Atropine-resistance and nerve-mediated ATP release. Atropine-resistance of nerve-mediated detrusor contractions is a well-established phenomenon in tissue from most small animals and pathological human bladders, but absent in the human-stable bladder. There is substantial, albeit mainly indirect, evidence that atropine-resistance results from nerve-mediated release of ATP, in addition to the normal secretion of acetylcholine (ACh). This is interpreted from the abolition of atropine-resistant contractions by ABMA, an agent which desensitises $P2X_1$ receptors (Palea *et al*., 1993; Bayliss *et al*., 1999). However, it must be appreciated that ABMA is also an agonist at other P2X receptor subtypes (Lê *et al*., 1998). ATP-dependent contractions are more rapid than those mediated by ACh and in animals rapid, partial urine voids are used territorial marking (Desjardins *et al*., 1973). However, there is equal interest regarding their presence in human storage and voiding pathologies and they may be responsible for overactive or non-voiding contractions. Apyrase is a highly active ATP-diphosphohydrolase and reduced nerve-mediated contractions by almost the same proportion that residual contractions were recorded in the presence of atropine. Thus, apyrase and atropine may reveal the same fraction of the contraction, namely that mediated by ATP. Some reports showed a small 6.5% purinergic component resistant to $P2X_1$ antagonists, assuming that ABMA primarily desensitises $P2X_1$ receptors, (Kennedy *et al*., 2007; Kennedy, 2015).

Causes of atropine-resistance. Several hypotheses were tested to account for the variable appearance of ATP-dependent nerve-mediated contraction in three cohorts: i) the potency and efficacy of detrusor for P2X receptors were different; ii) there was variable ATP release by the motor nerve; iii) ATP was hydrolysed to different extents in the neuromuscular

junction. The potency and efficacy of detrusor for ATP was tested in muscle strips and isolated myocytes from guinea-pig, human-stable and human-DO bladders, with no significant differences in muscle strips or in isolated cells. This functional observation in human detrusor is consistent with unchanged $P2X_1$ (O'Reilly *et al*., 2002) expression in detrusor from human pathological bladders, except an increase in obstructed bladders (O'Reilly *et al*., 2001). However, in rat tissue superfused in an organ bath for several hours there was a profound reduction of $P2X_1$ expression has been reported (Elliott *et al*., 2013), although in this study ABMA responses were stable over the experimental time-course. Another possibility is that ATP is not released from nerves in human-stable detrusor and is greatest in guinea-pig tissue. However, nerve-mediated ATP release was seen in all groups when ectoATPase activity was attenuated. Even though actual values may be attenuated by any remaining endogenous ectoATPase activity, it might argue against this being the principal cause for the lack of atropine-resistant contractions in human-stable detrusor.

The final possibility examined is that variable ectoATPase activity and expression of ATPases themselves accounts for the different extent of atropine-resistant contractions. EctoATPase activity was inversely associated with the magnitude of nerve-mediated purinergic contractions and this was corroborated in human tissue by reduced ectoATPase expression of the predominant enzyme ENTPDase-1 in detrusor from overactive compared to stable bladders. ENTPDase-1 is inhibited by ARL 67156 (Lévesque *et al*., 2007) which would account for the increase of nerve-mediated contractions in guinea-pig detrusor (Westfall *et al*., 1997) and validated its use to estimate ectoATPase activity in detrusor homogenates.

Variation of ectoATPase activity may also have an indirect effect through changing local concentrations of the products of ATP hydrolysis. ENTPDase-1 is the key subtype in human

detrusor (Silva-Ramos *et al*., 2015) which metabolises ATP to AMP, with further degradation to adenosine, but without intermediate accumulation of ADP. Thus, the absence of atropine-resistant contractions in human-stable detrusor may be contributed by greater degradation of nerve-mediated ATP release and also by adenosine itself attenuating ATP release via an A1-receptor mechanism (Pakzad *et al*., 2016). Adenosine reduced nerve-mediated contractions mostly at low frequencies, whilst ARL67156 increased contractions over the same frequency range making it difficult to distinguish between the two possibilities. Several arguments may favour variability of ectoATPase activity as an explanation for variable atropine-resistance: firstly nerve-mediated ATP release was similar in the three cohorts used in this study although atropine-resistance varied greatly; secondly the A1-receptor agonist N6-cyclopentyladenosine (CPA) had no effect on human-stable detrusor but a significant action on guinea-pig tissue (Pakzad *et al*., 2016) suggesting that adenosine-mediated suppression of ATP, *via* A1-receptors, was not a feature of human-stable detrusor.

Frequency-dependence of ATP release. Nerve-mediated detrusor contractions showing atropine-resistance have a component dependent on ATP, dominant at low frequencies, and another on ACh at higher frequencies and agrees with previous observations (Brading and Williams, 1990; Werner *et al*., 2007; Pakzad *et al*., 2016). Here we showed that direct measurement of nerve-mediated ATP release was over the same range of frequencies that generated purinergic contractions, in the presence of atropine. The differential frequency-dependence of ATP and ACh release conforms to the different cellular pathways that each transmitter regulates to generate contraction; a rapid P2X-dependent activation of myosin light chain kinase by Ca^{2+}-calmodulin and a slower muscarinic Ca^{2+}-desensitisation of contractile proteins (Tsai *et al*., 2012). A similar frequency-dependent release of ATP and

noradrenaline is present in ear artery and vas deferens preparations innervated by sympathetic nerves, with ATP release at lower frequencies (Kennedy *et al*., 1986; Todorov *et al*.,1996). The peak of the nerve-mediated ATP transient followed the tension transient (Figure 1A) which is counter-intuitive if ATP release generates force. However, Supplement 2 shows that this delay of the ATP response can be explained by delays in the ATP electrode response.

The ability to attenuate selectively nerve-mediated release of ATP rather than ACh offers the possibility of an interesting therapeutic target, as atropine-resistant contractions occur only in tissue from human-DO bladders. Adenosine, acting *via* an A1 receptor, and the PDE5 inhibitor sildenafil both attenuate the consequences of nerve-mediated ATP release (Searl *et al*., 2015; Pakzad *et al*., 2016; Chakrabarty *et al*., 2017). However, adenosine also reduces ACh release (Silva-Ramos et al., 2015) and the role of sildenafil in this context requires evaluation. A1 receptor agonists and PDE5 inhibitors respectively reduce cAMP or increase cGMP intracellular levels, both of which can attenuate activity of N-type or P/Q-type Ca^{2+} channels (Fukuda *et al*., 1996; Grassi *et al*; 2004; Nickels *et al*., 2007) that mediate Ca^{2+} influx necessary for vesicular neurotransmitter release. Of interest is that ω-conotoxin, an N-type blocker, but not ω-agatoxin, a P/Q-type blocker, attenuates nerve-mediated nucleotide release in human bladder (Breen *et al*., 2006). However, further studies are required to identify more clearly which Ca^{2+} channel subtype regulates vesicular ATP release and if they differ from ACh release pathways.

Limitations

The small size of the human biopsy samples precluded measurement of ENTPDase subtypes by Western blot as well as enable tension measurements with a separate strip. Thus, we

relied on qPCR to provide transcription data. ARL 67156 is a weak ENTPDase inhibitor, in particular the subtype most transcribed in detrusor, ENTPDase-1 (Lévesque *et al*., 2007). However, it may underestimate ectoATPase activity as a proportion of total tissue ATPase activity, it is assumed the proportional underestimation is similar in all preparation cohorts. In addition, it is assumed that ABMA desensitises $P2X_{1/3}$ receptors and not any other subtypes that may generate detrusor contractile activity. It has been suggested that with experiments using variable stimulation frequencies to elicit nerve-mediated contraction it is not the frequency *per se* but the number of stimuli that determines contraction magnitude. This has been addressed in Supplement 3, which shows that frequency of stimulation does indeed seem to be the relevant variable. Supplement 3 is an account of why the different stimulus parameters have been chosen to generate contractions.

Competing interests. The authors report no competing interests.

Declaration of transparency and scientific rigour. This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Authors' contributions.

- Devised the study: CHF, CMcC
- Contributed experiments: CMcC, YI, DS, BC, CHF
- Wrote drafts of the manuscript: CHF, CMcC, AJK, RIJ

- Approved the final manuscript: All authors

References

Alexander S. P. H., Christopoulos A., Davenport A. P., Kelly E., Marrion N. V., Peters J. A., … CGTP Collaborators (2017). The concise guide to PHARMACOLOGY 2017/18: G protein-coupled receptors. British Journal of Pharmacology, 174(Suppl 1), S17–S129. 10.1111/bph.13878

Alexander S. P. H., Fabbro D., Kelly E., Marrion N. V., Peters J. A., Faccenda E., … CGTP Collaborators (2017). The concise guide to PHARMACOLOGY 2017/18: Enzymes. British Journal of Pharmacology, 174(Suppl 1), S272–S359. 10.1111/bph.13877

Alexander S. P. H., Peters J. A., Kelly E., Marrion N. V., Faccenda E., Harding S. D., … CGTP Collaborators (2017). The concise guide to PHARMACOLOGY 2017/18: Ligand-gated ion channels. British Journal of Pharmacology, 174(Suppl 1), S130–S159. 10.1111/bph.13879

Bayliss M, Wu C, Newgreen D, Mundy AR, Fry CH (1999). A quantitative study of atropine-resistant contractile responses in human detrusor smooth muscle, from stable, unstable and obstructed bladders. *J Urol* **162**: 1833-1839.

Brading AF, Williams JH (1990). Contractile responses of smooth muscle strips from rat and guinea-pig urinary bladder to transmural stimulation: effects of atropine and alpha,beta-methylene ATP. *Br J Pharmacol* **99**: 493–498.

Breen LT, Smyth LM, Yamboliev IA, Mutafova-Yambolieva VN (2006). □-NAD is a novel nucleotide released on stimulation of nerve terminals in human urinary bladder detrusor muscle. *Am J Physiol Renal Physiol* **290**: F486-495.

Burnstock G, Cocks T, Crowe R, Kasakov L (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br J Pharmacol* **63**: 125-138.

Calvert RC, Thompson CS, Khan MA, Mikhailidis DP, Morgan RJ, Burnstock G (2001). Alterations in cholinergic and purinergic signaling in a model of the obstructed bladder. *J Urol* **166**: 1530-1533.

Chakrabarty B, Ito H, Ximenes M, Nishikawa N, Vahabi B, Kanai AJ, et al. (2019). Influence of sildenafil on the purinergic components of nerve-mediated and urothelial ATP release from the bladder of normal and spinal cord injured mice. *Br J Pharmacol*; **176**: 2227-2237.

Curtis MJ, Alexander S, Cirino G, Docherty JR, George CH, Giembycz MA *et al*. (2018). Experimental design and analysis and their reporting II: updated and simplified guidance for authors and peer reviewers. *Br J Pharmacol* **175**: 987–993.

Desjardins C, Maruniak JA, Bronson FH (1973). Social rank in house mice: differentiation revealed by ultraviolet visualization of urinary marking patterns. *Science* **182**: 939-941.

Elliott RA, Tonnu A, Ghaffar N, Taylor AH, Tincello DG, Norman RI (2013). Enhanced purinergic contractile responses and $P2X_1$ receptor expression in detrusor muscle during cycles of hypoxia-glucopenia and reoxygenation. *Exp Physiol*; **98**: 1683-1695.

Fukuda K, Kaneko K, Yada N, Kikuwaka M, Akaike A, Satoh M (1996). Cyclic AMP-dependent modulation of N- and Q-type Ca^{2+} channels expressed in Xenopus oocytes. *Neurosci Lett*; **217**: 13-16.

Grassi C, D'Ascenzo M, Azzena GB (2004). Modulation of Cav1 and Cav2.2 channels induced by nitric oxide via cGMP-dependent protein kinase. *Neurochem Int* **45**: 885-893.

Harding S. D., Sharman J. L., Faccenda E., Southan C., Pawson A. J., Ireland S., … NC-IUPHAR (2018). The IUPHAR/BPS guide to PHARMACOLOGY in 2018: Updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucl Acids Res, 46, D1091–D1106. 10.1093/nar/gkx1121

Harvey RA, Skennerton DE, Newgreen D, Fry CH (2002). Contractile potency of adenosine triphosphate and ecto-adenosine triphosphatase activity in guinea pig detrusor and detrusor from patients with a stable, unstable or obstructed bladder. *J Urol* **168**: 1235-1239.

Hashitani H, Suzuki H (1995). Electrical and mechanical responses produced by nerve stimulation in detrusor smooth muscle of the guinea-pig. *Eur J Pharmacol* **284**: 177-183.

Kennedy C. (2015) ATP as a cotransmitter in the autonomic nervous system. *Autonom Neurosci* **191**; 2-15.

Kennedy C, Saville VL, Burnstock G (1986). The contributions of noradrenaline and ATP to the response of the rabbit central ear artery to sympathetic nerve stimulation depend on the parameters of stimulation. *Eur J Pharmacol* **122**: 291-300.

Kennedy C, Tasker PN, Gallacher G, Westfall TD (2007). Identification of atropine- and $P2X_1$ receptor antagonist-resistant, neurogenic contractions of the urinary bladder. *J Neurosci* **27**: 845-851.

Kushida N, Fry CH (2016). On the origin of spontaneous activity in the bladder. *BJU Int* **117**: 982-992.

Lê KT, Babinski K, Seguela P (1998). Central P2X4 and P2X6 channel subunits coassemble into a novel heteromeric ATP receptor. *J Neurosci* **18**: 7152-7159.

Lee HY, Bardini M, Burnstock G (2000). Distribution of P2X receptors in the urinary bladder and the ureter of the rat. *J Urol* **163**: 2002-2007.

Lévesque SA, Lavoie EG, Lecka J, Bigonnesse F, Sévigny J (2007). Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases. *Br J Pharmacol* **152**: 141-150.

McGrath JC, Lilley E (2015). Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Br J Pharmacol* **172**: 3189–3193.

Montgomery BS, Fry CH (1992). The action potential and net membrane currents in isolated human detrusor smooth muscle cells. *J Urol* **147**: 176-84

Moss HE, Tansey EM, Burnstock G (1989). Abnormalities of responses to autonomic stimulation in the mouse urinary bladder associated with Semliki Forest virus-induced demyelination. *J Urol* **142**: 850-854.

Mumtaz FH, Lau DH, Siddiqui EJ, Morgan RJ, Thompson CS, Mikhailidis DP (2006). Changes in cholinergic and purinergic neurotransmission in the diabetic rabbit bladder. *In Vivo* **20**: 1-4.

Nickels TJ, Reed GW, Drummond JT, Blevins DE, Lutz MC, Wilson DF (2007). Does nitric oxide modulate transmitter release at the mammalian neuromuscular junction? *Clin Exp Pharmacol Physiol* **34**: 318-326.

North RA and Surprenant A (2000). Pharmacology of cloned P2X receptors. *Ann Rev Pharmacol Toxicol* **40**: 563-580

O'Reilly BA, Kosaka AH, Chang TK, Ford AP, Popert R, McMahon SB (2001). A quantitative analysis of purinoceptor expression in the bladders of patients with symptomatic outlet obstruction. *BJU Int* **87**: 617-622.

O'Reilly BA, Kosaka AH, Knight GF, Chang TK, Ford AP, Rymer JM *et al*. (2002). P2X receptors and their role in female idiopathic detrusor instability. *J Urol* **167**: 157-164.

Pakzad M, Ikeda Y, McCarthy C, Kitney DG, Jabr RI, Fry CH (2016). Contractile effects and receptor analysis of adenosine-receptors in human detrusor muscle from stable and neuropathic bladders. *Naunyn Schmiedebergs Arch Pharmacol* **389**: 921-299.

Palea S, Artibani W, Ostardo E, Trist DG, Pietra C (1993). Evidence for purinergic neuro-transmission in human urinary bladder affected by interstitial cystitis. *J Urol* **150**: 2007-2012.

Peterson JS, Noronha-Blob L (1989). Effects of selective cholinergic antagonists and alpha,beta-methylene ATP on guinea-pig urinary bladder contractions in vivo following pelvic nerve stimulation. *J Auton Pharmacol* **9**: 303-313.

Robson SC, Sévigny J, Zimmermann H (2006). The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. *Purinergic Signal* **2**: 409–430.

Searl TJ, Dynda DI, Alanee SR, El-Zawahry AM, McVary KT, Silinsky EM (2015). A1 adenosine receptor–mediated inhibition of parasympathetic neuromuscular transmission in human and murine urinary bladder. *J Pharmacol Exp Ther* **356**: 116–122

Silva-Ramos M, Silva I, Faria M, Magalhães-Cardoso MT, Correia J, Ferreirinha F *et al*. (2015). Impairment of ATP hydrolysis decreases adenosine A1 receptor tonus favoring cholinergic nerve hyperactivity in the obstructed human urinary bladder. *Purinergic Signal* **11**: 595-606.

Syed NH, Kennedy C (2012). Pharmacology of P2X receptors. *WIREs Membr Transp Signal* **1**: 16–30.

Todorov LD, Mihaylova-Todorova S, Craviso GL, Bjur RA, Westfall DP (1996). Evidence for the differential release of the cotransmitters ATP and noradrenaline from sympathetic nerves of the guinea-pig vas deferens. *J Physiol* **496**: 731-748

Tsai MH, Kamm KE, Stull JT (2012). Signalling to contractile proteins by muscarinic and purinergic pathways in neutrally-stimulated bladder smooth muscle. *J Physiol* **590**: 5107-5121.

Werner ME, Knorn AM, Meredith AL, Aldrich RW, Nelson MT (2007). Frequency encoding of cholinergic- and purinergic-mediated signaling to mouse urinary bladder smooth muscle: modulation by BK channels *Am J Physiol Regul Integr Comp Physiol* **292**: R616-624.

Westfall TD, Kennedy C, Sneddon P. (1997). The ecto-ATPase inhibitor ARL 67156 enhances parasympathetic neurotransmission in the guinea-pig urinary bladder. *Eur J Pharmacol* **329**: 169-173.

Wu C, Fry CH (2001). Na^+/Ca^{2+} exchange and its role in intracellular Ca^{2+} regulation in guinea pig detrusor smooth muscle. *Am J Physiol Cell Physiol* **280**: C1090-1096.

Yokota T, Yamaguchi O (1996). Changes in cholinergic and purinergic neurotransmission in pathologic bladder of chronic spinal rabbit. *J Urol* **156**: 1862-1866.

Yoshida M, Homma Y, Inadome A, Yono M, Seshita H, Miyamoto Y *et al* (2001). Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles. *Exp Gerontol* **36**: 99-109.

Young JS, Meng E, Cunnane TC, Brain KL (2008). Spontaneous purinergic neurotransmission in the mouse urinary bladder. *J Physiol* **586**: 5743-5755.

Zimmermann H, Zebisch M, Sträter N (2012). Cellular function and molecular structure of ecto-nucleotidases. *Purinergic Signal* **8**: 437–502.

Figure 1

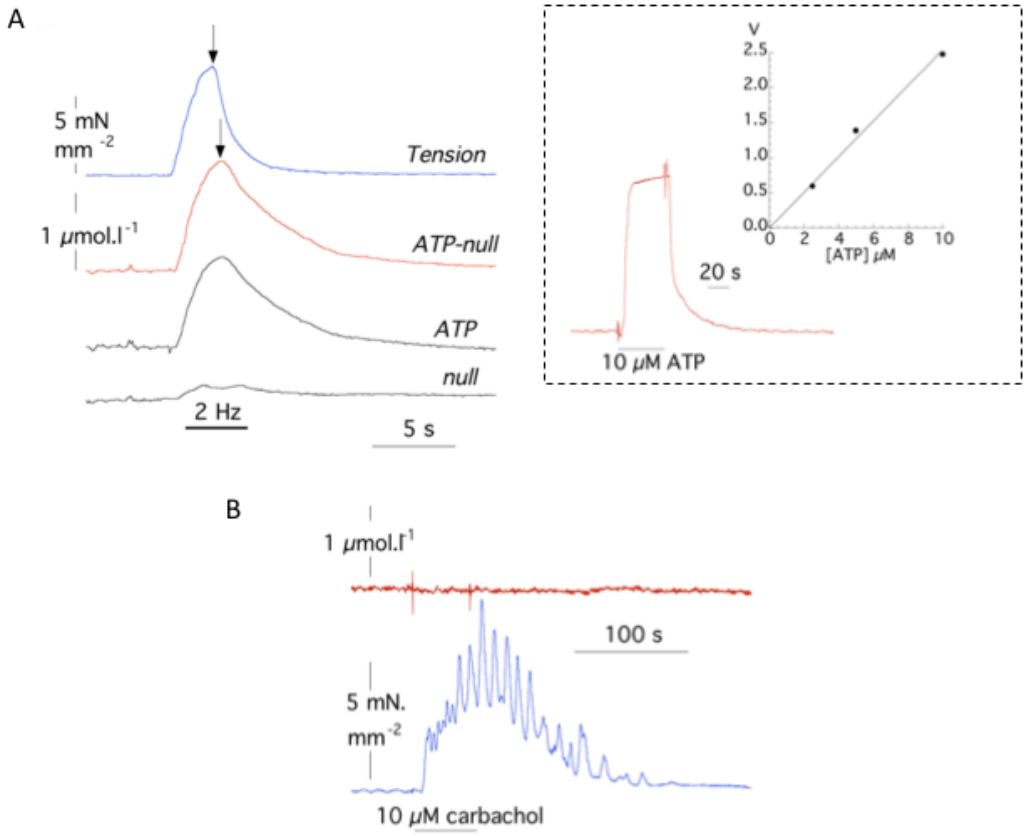

Figure 1

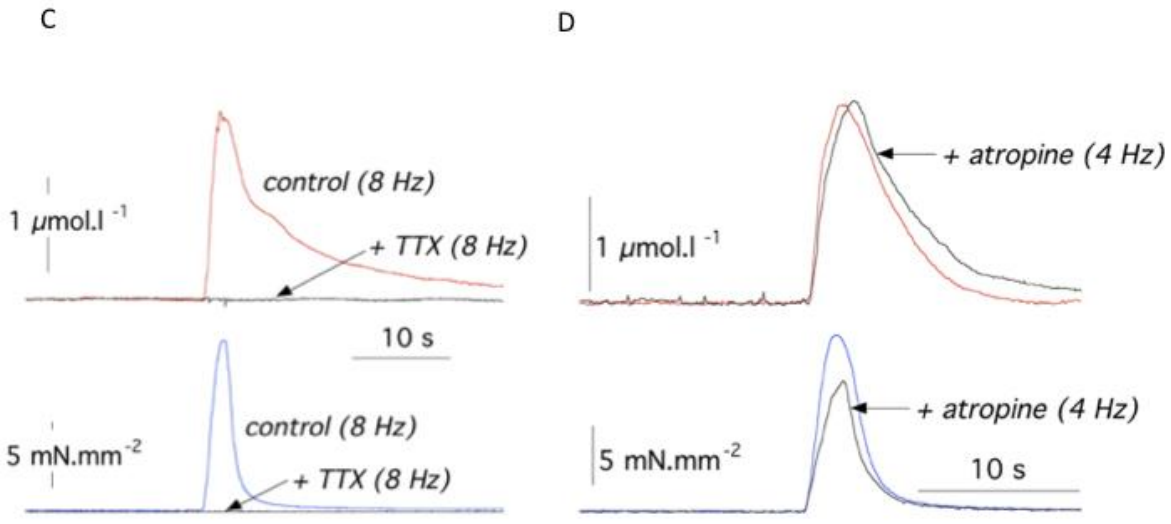

Figure 1: ATP transients in guinea-pig detrusor muscle preparations. A: Recordings of isometric tension (upper blue trace) and outputs from ATP-selective electrode and null electrodes (lower black traces), the difference recording (ATP-null, red trace) is also shown. Stimulation was a 3-s train at 2 Hz. Arrows above the tension and ATP-null traces show the respective times of peak values. The inset shows a calibration curve of an ATP electrode with a linear fit, as well as a sample calibration trace. B: Tension (blue) and ATP-null (red) traces in the presence of 10 μM carbachol. C: Tension (lower) and ATP-null (upper) traces in the absence and presence of 1 μM tetrodotoxin (TTX), in this example at 8 Hz stimulation. D: Tension (lower) and ATP-null (upper) traces in the absence and presence of 1 μM atropine, in this example at 4 Hz stimulation.

Figure 2

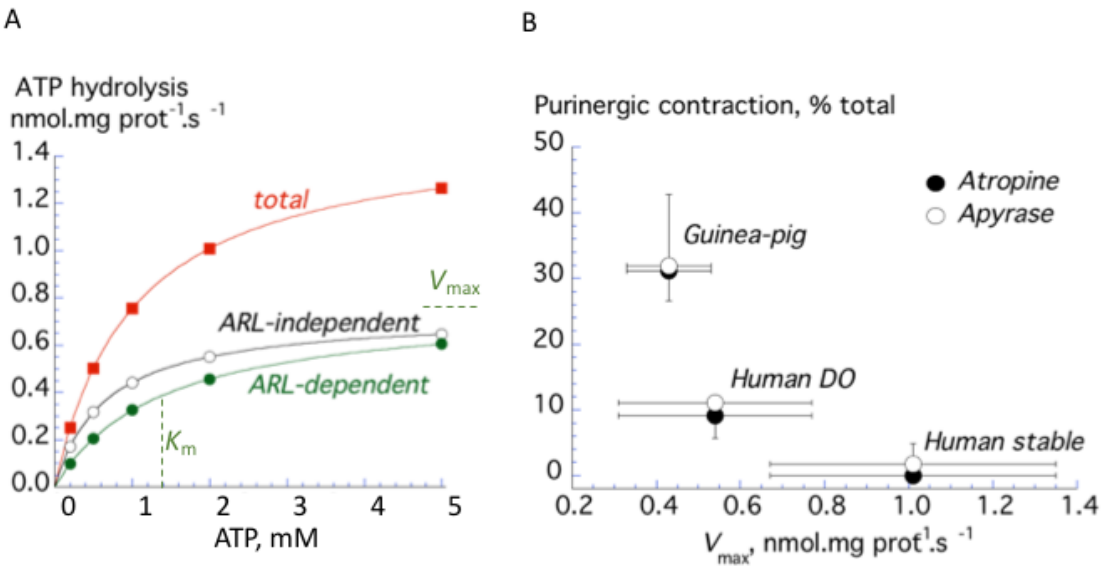

Figure 2. ecto-ATPase activity in detrusor smooth muscle. A: Sample experiment from human-stable detrusor tissue of the initial rate of ATP hydrolysis as a function of the starting ATP concentration, in the absence (total) and presence (ARL-independent) of 100 µM ARL 67156. The difference between the two (ARL-dependent) is also plotted as a measure of ecto-ATPase activity. The V_{max} and K_m values of the ARL-dependent fraction are shown. B: the relationship between ecto-ATPase V_{max} and the percentage purinergic component of the contraction (8 Hz stimulation) as determined by a) the percentage residual contraction with atropine (closed circles) or percentage reduction of the contraction by apyrase (open circles). Numbers of preparations in the three cohorts of tissue, contributing to the contractile data (ordinate) and ecto-ATPase data (abscissa), are shown in Table 1 and Supplement 1; Figures S1b, S1d and S1g.

Figure 3

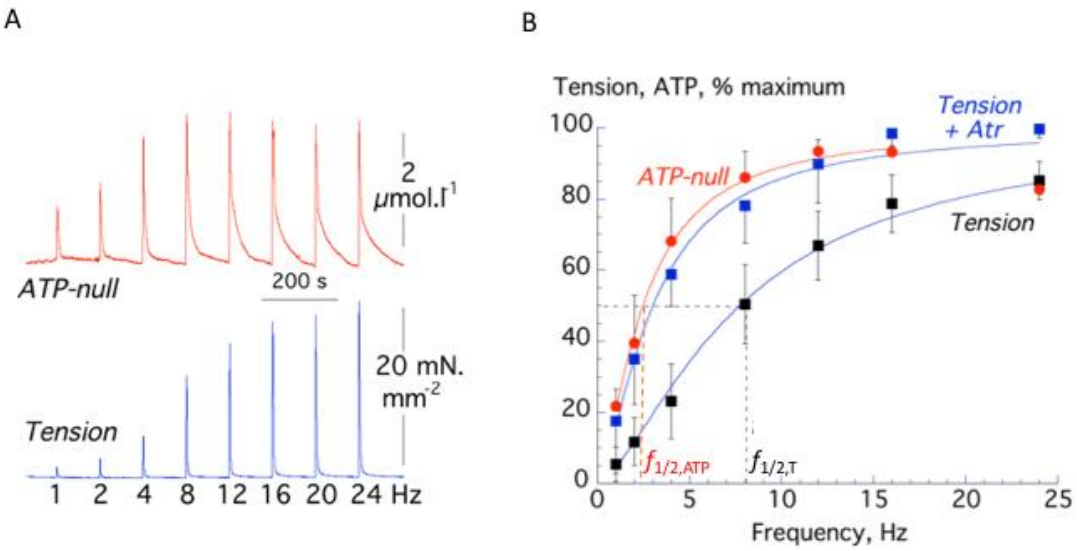

Figure 3. ATP-transients and nerve-mediated contractions. Data from guinea-pig preparations. A: Frequency-dependence of tension (lower, blue traces) and ATP-null (upper, red traces) traces. B: Dependence of the peak ATP transient (closed, red circles) and tension (black, closed squares) on stimulation frequency. Values of the half-maximal frequency ($f_{1/2}$) for tension (T, $f_{1/2,T}$) are shown. The mean value for the ATP-transient magnitude at 24 Hz was not used for the curve-fit. Also shown is the frequency-dependence of tension in the presence of 1 µM atropine (closed, blue squares). Data are mean ± SD, n=18 for tension values, n=6 for ATP data. See Supplement 1; Figure S1i, for $f_{1/2,T}$ and $f_{1/2,ATP}$ values in individual preparations.

Table 1. Values of ATP-dependent nerve-mediated force of contraction, responses to ABMA, ecto-ATPase activity and ENTPDase-1 expression. Number of preparations in parenthesis. Data are mean±SD, except for the atropine-resistance and apyrase-reduction data which are median [25,75% interquartiles] due to the skewed nature of some of these data sets. *p<0.05 *vs* human stable; $^{\S}p$<0.05 *vs* human overactive, $^{\#}p$<0.05 ABMA *vs* control. See figures S1-S8 in the Supplementary Information file for individual data points used to compile Table 1.

	Human stable	Human overactive	Guinea-pig
Nerve-mediated contractions			
Control, mN.mm^{-2} (8 Hz stim)	7.9 [6.6, 9.5] (19)	6.1 [3.6, 9,2] (16)	10.3 [8.5, 11.5] (7)
+atropine	0.0 [0.0, 0.08] (19)	0.6 [0.4, 1.2] (16)*	3.2 [2.8, 3.8] (7)*§
Atropine resistance, % total	0.0 [0.0, 1.0] (*19*)	9.1 [5.7, 29.9] (*16*)*	31.2 [26.6, 43.1] (*7*)*§
Control, mN.mm^{-2} (8 Hz stim)	8.5±2.6 (14)	11.2±4.7 (11)	11.4±3.3 (11)
+ABMA	7.4±2.6 (*14*)	7.3±4.4 (11)$^{\#}$	8.9±2.9 (11)$^{\#}$
Apyrase reduction, % control	1.7 [-1.1, 4.8] (*7*)	11.1 [5.7, 12.0] (*9*)*	31.9 [23.6, 42.8] (*9*)*§
ABMA, ATP potency and ABMA efficacy			
ABMA pEC_{50}, strips	5.51±0.11 (*7*)	5.41±0.12 (*7*)	5.53±0.13 (*7*)
ABMA pEC_{50}, myocytes	6.77±0.23 (*7*)	6.65±0.10 (*7*)	6.54±0.18 (*7*)
ABMA efficacy mN.mm^{-2}, strips	13.4±5.6 (*15*)	15.2±4.3 (*15*)	12.1±3.1 (*9*)
ATP, pEC_{50}	3.11±0.77 (*20*)	3.73±0.91 (*16*)*	3.86±0.40 (*8*)*
Ecto-ATPase activity			
V_{max}-total, nmol.mg^{-1}.s^{-1}	1.89±0.68 (*8*)	1.15±0.34 (*11*)	1.19±0.55 (*7*)
k_m-total, mM	1.46±0.30 (*8*)	1.38±0.26 (*11*)	1.07±0.15 (*7*)
V_{max} (ARL-sens), nmol.mg^{-1}.s^{-1}	0.98±0.25 (*8*)	0.60±0.16 (*11*)*	0.37±0.07 (*7*)*§
k_m (ARL-sens), mM	1.38±0.47 (*8*)	1.57±0.46 (*11*)	1.35±0.49 (*7*)
ENTPDase transcription			
ENTPDase-1/18S .10^{-4}	3.88±1.28 (*9*)	2.61±1.13 (*9*)*	
ENTPDase-2/18S .10^{-4}	0.035±0.023 (*9*)	0.029±0.027 (*9*)	
ENTPDase-3/18S .10^{-4}	0.090±0.070 (*9*)	0.047±0.050 (*9*)	
ENTPDase-5/18S .10^{-4}	0.11±0.05(*9*)	0.14±0.12 (*9*)	